PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SOPC/P28330PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB 03/01705	17.04.2003	17.04.2002
International Patent Classification (IPC) or both national classification and IPC		
C12M1/34		
Applicant		
SOPHION BIOSCIENCE A/S		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 13 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06.11.2003	18.06.2004
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Joyce, D Telephone No. +31 70 340-3093

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB 03/01705

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-7, 10, 11, 15-19, 21-36	as originally filed
8, 9, 12-14, 14a, 20, 20a	filed with telefax on 03.06.2004

Claims, Numbers

1-28	filed with telefax on 03.06.2004

Drawings, Sheets

1/11-11/11	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	26-28
Inventive step (IS)	Yes:	Claims	1-25
	No:	Claims	26-28
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	26-28

2. Citations and explanations

   see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: WO 00 71742 A (HICKMAN JAMES J) 30 November 2000 (2000-11-30)

D2: WO 01 25769 A (SOPHION BIOSCIENCE AS) 12 April 2001 (2001-04-12) cited in the application

D3: WO 99 31503 A (SCHMIDT CHRISTIAN ;VOGEL HORST (CH)) 24 June 1999 (1999-06-24)

D4: WO 01 48474 A (DODGSON JOHN ;ASTRAZENECA UK LTD (GB); ASTRAZENECA AB (SE)) 5 July 2001 (2001-07-05)

D5: SCHMIDT C ET AL: 'A CHIP-BASED BIOSENSOR FOR THE FUNCTIONAL ANALYSIS OF SINGLE ION CHANNELS' ANGEWANDTE CHEMIE. INTERNATIONAL EDITION, VERLAG CHEMIE. WEINHEIM, DE, vol. 39, no. 3137, 2000, pages 3137-3140, XP001002622 ISSN: 0570-0833

D6: DE 297 21 359 U (ITT IND GMBH DEUTSCHE) 12 February 1998 (1998-02-12)

1.0 Pior to assessing novelty, inventive step and industrial applicability of the claimed subject-matter it is noted that the claims contravene Rule 6.2(a) PCT, which clearly states that claims shall not contain references to the drawings &/or description.

1.1 The document D2 is regarded as being the closest prior art to the subject-matter of the independent claims 1, 15 and 24 and discloses (the references in parentheses applying to this document):
A substantailly planar substrate for use in patch clampanalysis of the electrophysiological properties of a cell membrane (cf D2 Page 9 line 4-7), wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane (cf D2 Fig:3C, Page 12 line 28-32).

1.2 Vis-a-vis this known device the apparatus of claim 1 and the method claims 15 and 24 differs in that the during measurment of the electrophyiological properties of cells consisting of a glycocalyx, the rim of the aperture protruding from the plane of the substrate to a height in excess of the thickness of the glycocalyx.

Hence the apparatus of claim1 and the corresponding method claims 15 and 24 are thus novel over the cited and consulted prior art.

1.3 This feature addresses the problem of overcoming the lack of integrity of the seal caused by the presence of a glycocalyx.

1.4 In particular, the rim is shaped such that the area of physical contact between the substrate and the cell membrane is minimised favouring penetration of the glycocalyx and formation of a non-compromised gigaseal. The presence of a rim as is recited in D2 would not have the effect of displacing the glycocalyx and thus the seal would be subsequently compromised . The fact that the rim exceeds the height of the glycocalyx ensures that the rim is equipped to penetrate the glycocalyx and promotes gigaohm seal formation.

1.5 Such an apparatus or method of making such an apparatus in which the rim must exceed the height of the glycocalyx is not apparent from the consulted or cited prior art, where this problem is not even mentioned.

1.6 Concluding the apparatus and method claims 1,15 and 24 respectively are also seen to involve an inventive step.
D1 does mention the formation of a gigaohm seal with cells containing a glycocalyx but fails to acknowledge the problems associated with the formation of such a seal for electrophysiological measurements, and also fails to disclose the rimmed aperture and subsequent modifications necessary to create a non-compromised gigaseal with such a cell possessing a glycocalyx.

1.7 As the industrial applicability of the subject-matter of claims 1, 15 and 24 is immediately apparent, all the requirements of Article 33 PCT are met.

1.8 Claims 2-14, 16-23, and 25 define further refinements of the inventive idea underlying claims 1, 15 and 24 respectively. As such these claims also meet the requirements of Article 33 PCT.

the substrate, which is turn is dependent on the size of the area of contact between the cell membrane and the substrate.

The physical area of contact between the cell membrane and a planar silicon chip (about 1μm width of contact rim; see Figure 2, right hand diagram) with a smoothly rounded, funnel-like orifice is much larger than that formed between a cell membrane and a glass micropipette (about 100 nm width; Figure 2, left hand diagram). This results in the force per unit area being considerably reduced in the chip relative to the pipette configuration, and the number of intercalated glycoproteins in the contact area being much larger, effectively preventing the required Angström distance between the phospholipid bilayer and the substrate surface imperative for the formation of a gigaseal.

The present invention seeks to address this problem by providing a planar substrate (e.g. a silicon-based chip), suitable for patch clamp studies of the electrophysiological properties of cell membrane, which is designed to provide a reduced area of contact with the cell membrane, thereby promoting the formation of a gigaseal.

Thus, a first aspect of the invention provides a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the rim protruding from the plane of the substrate to a height in excess of the thickness of the glycocalyx.

In a preferred embodiment, the substrate is a silicon-based chip.

In the present context, the term gigaseal normally indicates a seal of a least 1G ohm, and this is the size of seal normally aimed at as a minimum, but for certain types of measurements where the currents are large, lower values may be sufficient as threshold values.

By 'glycocalyx' we mean the layer created by the peptide- and glyco-moieties, which extend into the extracellular space from the glycoproteins in the lipid bilayer of the cell membrane.

Preferably, the rim extends at least 20nm, at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm or at least 100 nm above the plane of the substrate.

Advantageously, the rim is shaped such that the area of physical contact between the substrate and the cell membrane is minimised, thereby favouring penetration of the glycocalyx and formation of a gigaseal.

It will be appreciated by persons skilled in the art that the rim may be of any suitable cross-sectional profile. For example, the walls of the rim may be tapered or substantially parallel . Likewise, the uppermost tip of the rim may take several shapes, for example it may be dome-shaped, flat or pointed. Furthermore, the rim protrusion may be substantially perpendicular to, oblique, or parallel with the plane of the substrate. A parallel protruding rim may be located at or near to the mouth of the aperture or, alternatively, positioned deeper into the aperture. Conveniently, the width of the rim is between 10 and 200 nm.

It will be further appreciated by persons skilled in the art that the aperture should have dimensions which do not permit an intact cell to pass through the planar substrate.

Examples of the general design of the preferred embodiment of the first aspect of the invention wherein the substrate comprises integral electrodes (but without the rimmed aperture feature of the present invention) are described in WO 01/25769.

A second aspect of the invention provides a method of making a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the method comprising the steps of:

(i) providing a substrate template;

(ii) forming an aperture in the template; and

(iii) forming a rim around the aperture such that the rim protrudes from the substrate to a height in excess of the thickness of the glycocalyx.

Preferably, the substrate is manufactured using silicon micro fabrication technology "Madou, M., 2001".

It will be appreciated by persons skilled in the art that steps (ii) and (iii) may be performed sequentially (i.e. in temporally separate steps) or at the same time.

Advantageously, step (ii) comprises forming an aperture by use of an inductively coupled plasma (ICP) deep reactive ion etch process. "Laermer F. and Schilp, A., DE4241045"

When it is required to form a substantially vertical protrusion relative to the plane of the substrate, the method comprises an intermediate step of a

directional and selective etching of the front side of the substrate causing a removal of a masking layer on the front side of the substrate, and further proceeding the prescribed protrusion distance into the underlying substrate.

As a result of a faster etch rate of silicon compared to that of the masking material, the masking material will be left inside the aperture, and protrude from the surface. An overall surface coating can subsequently be applied.

When it is required to form a protrusion lying substantially in the plane of the substrate, the method comprises an intermediate step of using Inductively Coupled Plasma (ICP) etch or Advanced Silicon Etch (ASE) for the formation of the pore, where the repetitive alternation of etching and passivation steps characterising these methods, will result in some scalloping towards the mouth of the aperture. By suitable adjustment of the process parameters, the scalloping can result in an inward in plane protrusion of the rim.

Again, an overall surface coating can subsequently be employed.

Conveniently, the method further comprises coating the surface of the substrate (e.g. with silicon oxide), either before or after formation of the aperture and/or rim. Alternatively, step (iii) is performed at the same time as coating the substrate.

Such coatings may be deposited by use of plasma enhanced chemical vapour deposition (PECVD) and/or by use of low pressure chemical vapour deposition (LPCVD).

The preferred embodiment of the first aspect of the invention wherein the substrate comprises integral electrodes may be manufactured as described in WO 01/25769).

A third aspect of the invention provides a method for analysing the electrophysiological properties of a cell membrane comprising a glycocalyx, the method comprising the following steps:

(i) making a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the method comprising the steps of

(ii) providing a substrate template;

(iii) forming an aperture in the template; and

(iv) forming a rim around the aperture such that the rim protrudes from the substrate to a height in excess of the thickness of the glycocalyx.

(v) contacting the cell membrane with the rim of an aperture of the substrate such that a gigaseal is formed between the cell membrane and the substrate; and

(vi) measuring the electrophysiological properties of the cell membrane.

In a preferred embodiment of the third aspect of the invention, there is provided a method of establishing a whole cell measuring configuration for determining and/or monitoring an electrophysiological property of one or more ion channels of one or more ion channel-containing structures, said method comprising the steps of:

(i) providing a substrate as defined above;

(ii) supplying a carrier liquid at one or more apertures, said carrier liquid containing one or more ion channel-containing structures;

(iii) positioning at least one of the ion channel-containing structures at a corresponding number of apertures;

(iv) checking for a high electrical resistance seal between an ion channel-containing structure held at a site (i.e. aperture) and the surface part of the site (i.e. rim) with which the high electrical resistance seal is to be provided by successively applying a first electric potential difference between the measuring electrode associated with the site and a reference electrode, monitoring a first current flowing between said measuring electrode and said reference electrode, and comparing said

provided. The person skilled in the art will be able to select such suitable measuring circuit.

A fourth aspect of the invention provides a kit for performing a method according to Claim 24, the kit comprising a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the rim protruding from the plane of the substrate to a height in excess of the thickness of the glycocalyx and one or more media or reagents for performing patch clamp studies.

Preferably the kit comprises a plurality of substrates.

The invention will now be described with reference to the following non-limiting examples and figures:

Figure 1 shows the cell with a patch pipette attached. In the gigaseal zone, (indicated by shaded area at point of contact between the pipette tip and the cell membrane) the glycoproteins of the glycocalyx have been displaced laterally to allow direct contact between the membrane phospholipid bilayer and the pipette;

Figures 2a and 2b show a cell attached to either a pipette tip (Figure 2a) or a planar substrate (Figure 2b). The area of contact between the cell membrane and substrate surface is considerably larger in the substrate configuration (Figure 2b) than in the pipette configuration (Figure 2a).

Figure 3 shows the variation in actual pipette resistance for each intended resistance set;

Figure 4 shows Gigaseal success rate versus pipette resistance;

Figure 5 shows the success rate of whole-cell establishment (from successful gigaseals) versus pipette resistance;

Figure 6 shows the time-dependence of gigaseal formation with different aperture sizes, the error bars indicating the standard deviation from the mean;

CLAIMS

1. A substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the rim protruding from the plane of the substrate to a height in excess of the thickness of the glycocalyx.

2. A planar substrate according to Claim 1 wherein the rim protrudes from the plane of the substrate to a height of at least 20 nm above the surface of the planar substrate, preferably least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm or at least 100 nm.

3. A planar substrate according to any one of the preceding claims wherein the width of the rim is in the range 50 to 200 nm.

4. A planar substrate according to any of the preceding claims, in which the length (i.e. depth) of the aperture is between 2 and 30 μm, preferably between 2 and 20 μm, 2 and 10 μm, or 5 and 10 μm.

5. A planar substrate according to any of the preceding claims wherein the diameter of the aperture is in the range 0.5 to 2 μm.

6. A planar substrate according to any one of the preceding claims wherein the rim extends substantially perpendicularly to the plane of the substrate.

7. A substrate according to any one of Claims 1 to 5 wherein the rim forms an oblique angle with the plane of the substrate.

8. A substrate according to any one of Claims 1 to 5 wherein the rim is substantially parallel to the plane of the substrate.

9. A substrate according to Claim 1 wherein the rim is defined by a mouth of the aperture, which mouth has a radius of curvature between 5 and 100nm with an angle of 45 to 90 degrees.

10. A planar substrate according to any of the preceding claims wherein the substrate is made of silicon, plastics, pure silica or other glasses, such as quartz and PyrexTM, or silica doped with one or more dopants selected from the group of Be, Mg, Ca, B, Al, Ga, Ge, N, P, As.

11. A planar substrate according to Claim 10 wherein the substrate is made of silicon.

12. A substrate according to any of the preceding claims wherein the surface of the substrate and/or the walls of the aperture are coated with a second coating material.

13. A substrate according to Claim 12 wherein the coating material is silicon, plastics, pure silica, other glasses such as quartz and PyrexTM, silica doped with one or more dopants selected from the group of Be, Mg, Ca, B, Al, Ga, Ge, N, P, As, or oxides of the same.

14. A substrate according to Claim 10 wherein the coating material is silicon oxide.

15. A method of making a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the method comprising the steps of

(i) providing a substrate template;

(ii) forming an aperture in the template; and

(iii) forming a rim around the aperture such that the rim protrudes from the substrate to a height in excess of the thickness of the glycocalyx.

16. A method according to Claim 15 wherein the substrate is manufactured using silicon micro fabrication technology.

17. A method according to Claims 15 or 16 wherein step (ii) comprises forming an aperture by use of an inductively coupled plasma (ICP) deep reactive ion etch process.

18. A method according to any one of Claims 15 to 17 further comprising the step of coating the surface of the substrate.

19. A method according to Claim 18 wherein step (iii) is performed at the same time as coating the substrate.

20. A method according to Claim 18 wherein step (iii) comprises an intermediate step of a directional and selective etching of the front side of the substrate causing a removal of a masking layer on the front side of the

substrate, and further proceeding the prescribed protrusion distance into the underlying substrate.

21. A method according to Claims 18, 19 or 20 wherein the coating is deposited by use of plasma enhanced chemical vapour deposition (PECVD) and/or by use of low pressure chemical vapour deposition (LPCVD).

22. A method according to Claim 21 wherein the coating is deposited by use of plasma enhanced chemical vapour deposition (PECVD).

23. A method according to Claim 17 wherein step (iii) comprises forming a rim from a deposited surface coating by use of plasma enhanced chemical vapour deposition (PECVD).

24. A method for analysing the electrophysiological properties of a cell membrane comprising a glycocalyx, the method comprising the following steps:

(i) making a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the method comprising the steps of

(ii) providing a substrate template;

(iii) forming an aperture in the template; and

(iv) forming a rim around the aperture such that the rim protrudes from the substrate to a height in excess of the thickness of the glycocalyx.

(v) contacting the cell membrane with the rim of an aperture of the substrate such that a gigaseal is formed between the cell membrane and the substrate; and

(vi) measuring the electrophysiological properties of the cell membrane.

25. A kit for performing a method according to Claim 24, the kit comprising a substantially planar substrate for use in patch clamp analysis of the electrophysiological properties of a cell membrane comprising a glycocalyx, wherein the substrate comprises an aperture having a rim defining the aperture, the rim being adapted to form a gigaseal upon contact with the cell membrane, the rim protruding from the plane of the substrate to a height in excess of the thickness of the glycocalyx and one or more media or reagents for performing patch clamp studies.

26. A substrate substantially as herein before described with reference to the accompanying drawings.

27. A method substantially as herein before described with reference to the accompanying drawings.

28. A kit substantially as herein before described with reference to the accompanying drawings: